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(Progress Report)

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

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I. INTRODUCTION

Until recently mosquito repellents have been evaluated mainly on humans utilizing subjective criteria. However, human bioassay virtually excludes testing of internally administered compounds as well as testing of many subjects. If new compounds are to be studied as surface or as internally administered repellents, the tests must be conducted on suitable laboratory animals. The objective of the current phase of the program was to develop a screening method for mosquito repellency by using mice. The method developed was tested with commercially available repellents and was found to be a reliable screening technique.

II. PRELIMINARY WORK

Adult mosquitoes of strain Aedes aegypti were cultured in the laboratory according to the method described in the first report, (ARF 3222-1). The pupae were sorted according to sex and placed in separate cages. The adult female mosquitoes were anesthetized with ether, and 50 were transferred to dry cages which were provided with a tube in the center containing water. All the cages were stored in a constant environment of 29° C with relative humidity about 85%. A cotton gauze pad soaked with 10% sucrose solution was placed on the cover of each cage. The covers of the cages consisted of nylon netting, through which the mosquitoes were allowed to feed on the surcrose. The mosquitoes were starved for 48 hr before testing.

A. Screening by Fluorescent Indicator

Blanchophor is a fluorescent dye which binds with plasma proteins and thus indicates the presence of plasma proteins. Intravenous injection of 200 mg/kg of this dye did not produce death in 4 test mice.

In the bioassay procedure 100 mg/kg (aqueous solution) of Blancophor was injected intravenously into mice 5 min before they were exposed to the mosquitoes. Abdomens of the mosquitoes which had fed on mice treated with Blancophor were bright green when illuminated with ultraviolet light, while those that had not fed remained black. Percent repellency was determined as follows:

$$\text{Percent repellency} = \frac{\text{Number without Blancophor}}{\text{Total number of mosquitoes}} \times 100$$

As indicated in Table 1, the Blancophor test proved to be useful and reliable for detecting the mosquitoes which ingested mouse blood. A spray of "Off," a commercially available mosquito repellent, completely prevented biting. The dye itself did not show any repellency. Male mosquitoes, which are known not to suck blood, could be easily distinguished from females. We are currently quantitating the amount of plasma bound dye sucked by each mosquito.

Table 1

MOSQUITO REPELLENCY AS MEASURED BY BLANCOPHOR INDICATOR IN MICE

Repellent	Mosquitoes Tested, number Female	Male	Fluorescent Mosquitoes, number	Engorged Mosquitoes, %
None	50	0	50	100
None	50	10	50	80
Off	50	0	0	0

B. Screening by Radioactive Indicators

It is well known that the mode as well as the degree of repellency differ from one compound to another. It is therefore imperative to determine the degree of repellency along with the total absence of biting. The obvious approach to this problem is to determine the amount of blood a mosquito ingests during sucking. Since the amount of blood sucked by a mosquito is very small, the use of radiotracers was considered as a quantitative measure of this amount.

Several radio active elements have been used before for this purpose. P^{32}-labeled phosphoric acid used in vitro is not suitable for in vivo use because it is rapidly removed from the blood and is incorporated in the tissues or excreted from the body. Therefore, its level cannot be maintained constant for any length of time. Moreover, the P^{32} contained in the excreta of mosquitoes may contaminate the other mosquitoes in the cage.

Booeman has injected Ce^{144} into mice in order to estimate the amount of blood ingested by insects.[1] This isotope remains in the blood for a longer period than P^{32}. However, the high cost, the affinity for bone tissue, and the health hazards associated with the use of this isotope reduce its suitability for routine use.

Urea has been used to measure water spaces in body organs, and we explored the possibility of using this isotope as an indicator. Mice were injected intravenously with 10 μc of urea-C^{14} and the distribution of the agent was observed 2 and 60 min after injection by the technique of whole-animal autoradiography. As seen in Fig. 1, urea concentrated in the skin. This feature makes it a good indicator. However, the level of radioactivity declined within 1 hr after injection.

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[1] Booeman, J. P. T., Ann. Trop. Med. and Parasitol., 54, 8, 1960.

Figure 1

DISTRIBUTION OF UREA-C^{14} IN A MOUSE
2 MIN AFTER INTRAVENOUS INJECTION

To determine whether internally administered urea has any repellency, two levels of urea (25 mg/kg and 2, 500 mg/kg) were administered intraperitoneally to mice. When exposed to starved female mosquitoes, these mice attracted the insects to the same degree as noninjected mice.

To determine the amount of radioactivity a mosquito ingests during the experimental test period, 10 μc of urea-C^{14} was injected intravenously to a mouse and the mouse was exposed to 50 starved female mosquitoes. The radioactivity ingested by the mosquitoes was less than 0.0001% of the total radioactivity injected.

After these preliminary experiments the possibility of using urea as an indicator was abandoned, for several reasons. First, because of the low radioactivity found in the mosquitoes, a higher dose of isotope would be required in the mice. Second, it is likely that mosquitoes contain urease, which might hydrolyze the urea before it is measured. Third, the radioactivity in the mosquitoes declined within the experimental period.

Another approach to the problem is to measure the amount of albumin ingested by mosquitoes. This can be accomplished by using radioactive serum albumin (RISA-I^{131}). Previous studies have established that the level of RISA-I^{131} in the blood remains constant for a long period of time. This observation was confirmed in our laboratories. A mouse was injected intravenously with RISA-I^{131}, and duplicate blood samples were drawn from the orbital cavity at several time intervals and the radioactivity measured. The data in Table 2 show a constant level of RISA-I^{131} in the blood for periods as long as 1 hr after injection.

Table 2

LEVEL OF RISA-I^{131} IN THE BLOOD OF A MOUSE AFTER INTRAVENOUS INJECTION

Time after Injection, min	Radioactivity, counts per minute per 10 µl of blood
12	7880
22	9267
32	8632
42	7811
52	6807

RISA-I^{131} is limited to the vascular compartment of mice after intravenous administration. This was shown by whole-animal autoradiograms prepared from several mice each injected with RISA-I^{131} and sacrificed at various time intervals. A typical autoradiogram is given in Fig. 2. It shows the presence of radioactivity in blood vessels only.

Since RISA-I^{131} is confined to the vascular bed for the experimental period its concentration in the blood ingested by mosquitoes should be directly proportional to the dose of RISA-I^{131} injected. This is evident from the data in Table 3. Since the amount and composition of blood ingested by mosquitoes is constant, the radioactivity found is directly porportional to the dose of RISA-I^{131}.

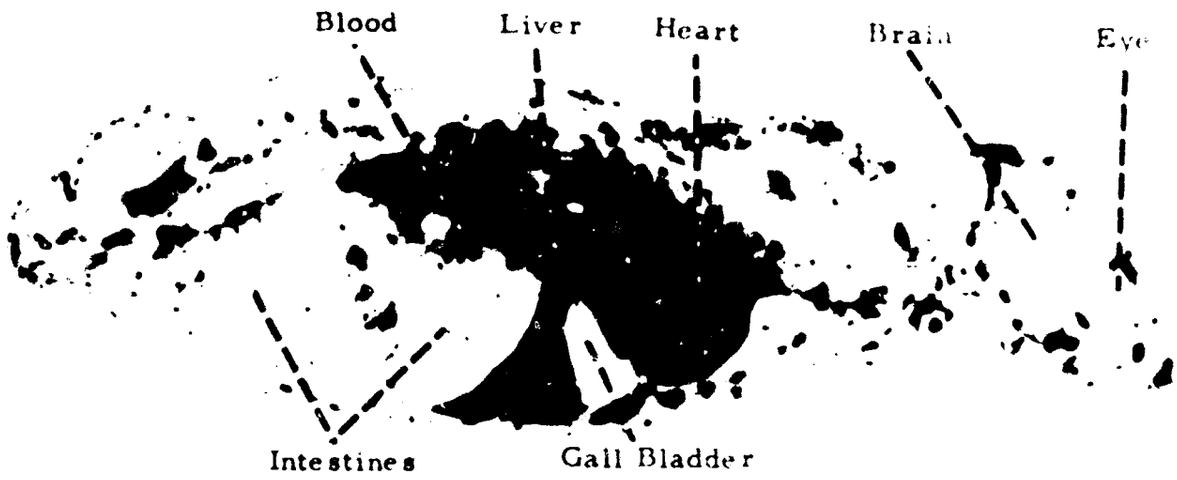

Figure 2

DISTRIBUTION OF RISA-I^{131} IN A MOUSE
5 MIN AFTER INTRAVENOUS INJECTION

Table 3

AMOUNT OF RISA-I^{131} INGESTED BY FEMALE MOSQUITOES FEEDING ON MICE INJECTED WITH RISA-I^{131}

Dose of RISA-I^{131}, μc	Radioactivity, counts per minute per 50 mosquitoes
4	80, 525
8	159, 442

To test its reliability as an indicator, 0.1 ml of RISA-I^{131} was injected into 9 mice, 4 of which were sprayed with "Off" 5 min before injection. The other 5 served as controls. The estimated radioactivity in the female mosquitoes feeding on the mice for 30 min is given in Table. 4. The mice treated with "Off" sucked negligilible amount of blood as compared to the control group. The results show good reliability and reproducibility.

Table 4

AMOUNT OF RISA-I^{131} INGESTED BY FEMALE MOSQUITOES FEEDING ON REPELLENT-TREATED AND UNTREATED MICE INJECTED WITH RISA-I^{131}

Mice	Radioactivity, counts per minute per 50 mosquitoes	Mean Weight per 50 Mosquitoes after Feeding, mg
Untreated	656, 896	199.3
	748, 694	202.9
	951, 608	230.9
	486, 350	224.3
	837, 573	198.1
	860, 594	236.7
Treated with OFF	147	127.5
	231	122.1
	198	110.1
	217	

Our observations show that RISA-I^{131} is a suitable indicator and fulfills the necessary requirements for the screening program. Therefore, it was employed in all subsequent experiments.

C. Screening by Weight Increase

Some investigators have utilized the increase in the weight of mosquitoes after feeding as a measure of ingested blood. We explored the possibility of using this approach for bioassay. Table 4 and 5 lists the weights of mosquitoes before and after feeding on normal and repellent-treated mice. It is seen that determination of weight gain is a reliable indicator of all-or-none types of observation. Subsequent experiments showed that weight gain is not a reliable measurement of partial repellency. However, we continued to measure weight as well as radioactivity in the bioassay.

Table 5

WEIGHT OF FEMALE MOSQUITOES BEFORE AND AFTER FEEDING ON REPELLENT-TREATED AND UNTREATED MICE

Mice	Number of Experiments	Mean Weight per 50 Mosquitoes, mg + S.E. Before Feeding	After Feeding
Untreated	15	124.9 ± 1	230.1 ± 5.2
Treated	3	120.2	120.2

III. FINAL BIOASSAY PROCEDURE

A prospective repellent is administered to a mouse by an appropriate route at a predetermined time prior to testing. The mouse is given an interperitoneal injection of Nembutal (80 mg/kg) 10 min before exposure. Five minutes before testing RISA-I^{131} solution (0.005 ml/gm) is injected intravenously. The mouse is then placed flat on its abdomen on the netting covering the mosquito cage. The cage contains 50 female mosquitoes which have been starved for 48 hr. The mouse is left on the cage for 30 min (biting period), after which time the mosquitoes are killed with ether and immediately weighed. They are then measured for radioactivity in the scintillation counter (Nuclear Chicago).

Duplicate samples of 10 µl of blood are withdrawn into a micropipette by an orbital bleeding technique, described by Riley.[2] The fragile capillaries of the ophthalmic venous plexus which lines the back of the orbit are ruptured by contact with a heparinized capillary, and a 10 µl blood sample is withdrawn. The blood is measured for radioactivity in the scintillation counter.

The amount of blood sucked by 50 female mosquitoes is calculated by the following formula:

$$\mu l \text{ of blood} = \frac{C\ P\ M/50 \text{ Mosquitoes}}{C\ P\ M/\mu l \text{ of blood}}$$

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[2] ley, V. Proc. Soc. Expt. Biol. Med., 104, 751, 1960.

IV. SCREENING TESTS WITH REPELLENTS

A new, untested compound and a number of known surface repellents previously tested on humans were tested by the RISA-I^{131} bioassay technique. Swiss albino male mice weighing 25 to 30 g were used as bait. Acetone and ethyl alcohol were used as solvents for water-insoluble compounds. Table 6 shows quantitative data from these experiments. It is seen that these solvents did not decrease the attraction of the bait, but the topically applied chemicals completely prevented biting.

The new, untested compound is allethrine, which proved to be a highly effective repellent. A 1% allethrine-acetone solution completely prevented mosquito biting 5 min and 30 min after application. Allethrine was originally synthesized as a substitute of pyrethrine, a potent insecticide. Allethrine is said to be more toxic to insects than pyrethrine, but it is less toxic to mammals. Low oral as well as percutaneous toxicity of allethrine has been reported in literature. The intraperitoneal toxicity of allethrine was determined during the present investigation. The approximate LD_{50}, as calculated from the data shown in Fig. 3 according to the method of Litchfield and Wilcoxon is 155 mg/kg.[3]

Since the major objective of the current investigation is the development of an effective orally administered mosquito repellent, allethrine was tested for its systemic efficacy. Allethrine was injected intraperitoneally as an aqueous suspension using 0.001% Tween 80 as the emulsifying agent. Injection of

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[3] Litchfield, J. T. and F. Wilcoxon, J. Pharmacol. Expt. Therap., 96, 99, 1949.

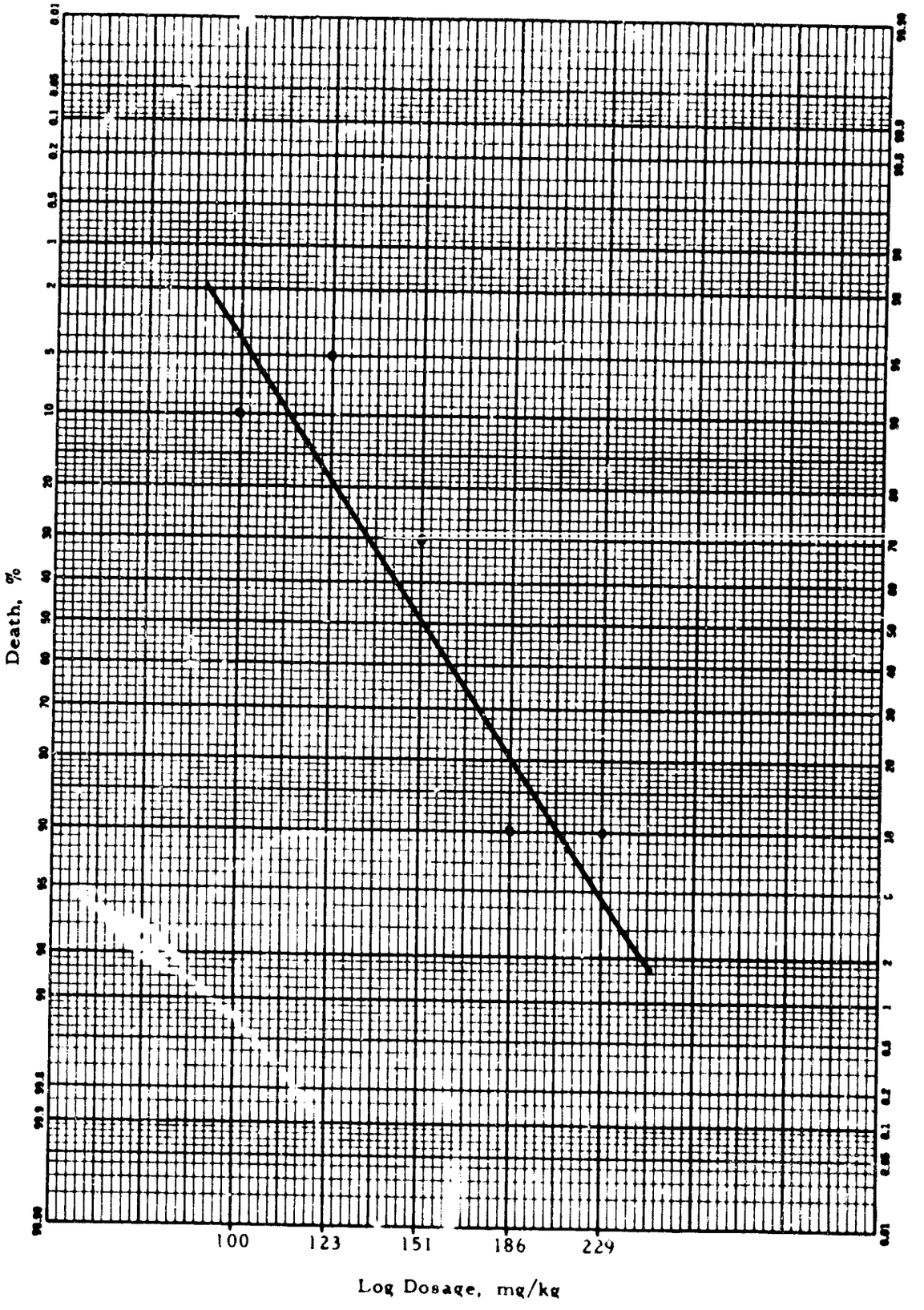

Figure 3

ACUTE TOXICITY OF ALLETHRINE (I.P.) IN MICE

Table 6

REPELLENCY OF VARIOUS TOPICALLY APPLIED COMPOUNDS

Repellent,	% w/v	Number of Experiments	Mean Weight per 50 Female Mosquitoes, after Feeding mg ± S.E.	Blood Ingested per 50 Mosquitoes after Feeding µl ± S.E.
None		14	230 ± 5.2	
None		9		1682. ± 17.3
Acetone	100	1	232.5	208
Ethyl alcohol	100	1	241.4	270
Indalone	50	1	93.4	0
N, N-Diethyl-m-toluamide	50	1	108.6	0
N, N-Propylacetanilide	50	1	110.5	0
Decanoic acid	50	1	102.1	4.9
10-Undecanoic acid	50	1	114.5	26.4
Citronellic acid	50	1	132.1	11.2
Benzyl benzoate	50	1	130.2	21.1
Anisyl alcohol	50	1	107.9	15.3
2-Ethyl-1, 3-hexanediol	50	1	159.8	46.4
Allethrine	100	1		3.0
Allethrine	20	1		0
Allethrine	1	4	99.7	0
Allethrine	1*	4	106.2	0

* 30 min after local application; all other tests were made 5 min after.

allethrine 15 min prior to testing reduced the amount of blood ingested by 50%, 2 hrs after injection the amount of blood ingested was reduced by 66%. Subcutaneous administration of allethrine prevented mosquito biting completely. These results are shown in Table 7.

The repellency shown by subcutaneous administration may or may not be a real effect. Since allethrine is a potent surface repellent, any contamination with allethrine during the subcutaneous injection may be effective in repelling mosquitoes. But the effect of interperitoneal administration is significant and will be pursued further.

Table 7

REPELLENT PROPERTIES OF INTERNALLY ADMINISTERED ALLETHRINE

Dose, mg/kg	Route of Administration	Number of Experiments	Time after Injection, min	Mean Weight per 50 female mosquitoes after Feeding, mg + S.E.	Microliters of blood meal Mean + S.E.
0		14	---	230.1 ± 5.2	168.3[1] ± 17.3
100	Subcutaneous	2	80	129.7	1
330	Intraperitoneal	1	40	----------	0
100	Intraperitoneal	4	5	164.9	110.5 ± 28.4
100	Intraperitoneal	4	15	149.5	73.5 ± 26.5
100	Intraperitoneal	4	60	154.2	72.0 ± 23.5
100	Intraperitoneal	4	120	136.5	58.1 ± 5.4

[1] Mean of 9 experiments.

V. FUTURE WORK

Additional compounds will be tested for mosquito repellency by the RISA-I^{131} bioassay technique. Simultaneously, physiochemical properties of nearly 100 compounds will be determined. The promising mosquito repellents will be tested for toxicity to the mice.

VI. RECORDS

All experimental data are recorded in ARF Logbook No. C 12516.

Respectfully submitted,

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